

U.S.S.N. 10/086,745  
DE JONG, *et al.*  
PRELIMINARY AMENDMENT

(DOPE), 2,3-dioleoyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA),  $C_{52}H_{106}N_6O_4 \cdot 4CF_3CO_2H$ ,  $C_{88}H_{178}N_8O_4S_2 \cdot 4CF_3CO_2H$ ,  $C_{40}H_{84}NO_3P \cdot CF_3CO_2H$ ,  $C_{50}H_{103}N_7O_3 \cdot 4CF_3CO_2H$ ,  $C_{55}H_{116}N_8O_2 \cdot 6CF_3CO_2H$ ,  $C_{49}H_{102}N_6O_3 \cdot 4CF_3CO_2H$ ,  $C_{44}H_{89}N_5O_3 \cdot 2CF_3CO_2H$ ,  $C_{41}H_{78}NO_8P$ ,  $C_{100}H_{206}N_{12}O_4S_2 \cdot 8CF_3CO_2H$ ,  $C_{162}H_{330}N_{22}O_9 \cdot 13CF_3CO_2H$ ,  $C_{43}H_{88}N_4O_2 \cdot 2CF_3CO_2H$ ,  $C_{43}H_{88}N_4O_3 \cdot 2CF_3CO_2H$  and (1-methyl-4-(1-octadec-9-enyl-nonadec-10-enylenyl) pyridinium chloride.

REMARKS

Any fees that may be due in connection with filing this paper, or during the entire pendency of this application, may be charged to Deposit Account No. 50-1213.

Claims 1-32 are pending in this application. Claims 1, 9-11, 21 and 27 are amended. Claim 1 is amended to add the inadvertently omitted claim element labels "(a)" through "(c)". The claim element labels in claim 1 are necessary to clarify claim element references made in claims dependent thereon. Claim 9 is amended to correct improper claim dependency of the claim. Claim 1 provides antecedent basis for introducing labeled nucleic acid molecules that encode a reporter gene into cells. Hence, claim 9, which further expands upon the description of the labeled nucleic acid molecules, first introduced in claim element (a) of claim 1, should depend on claim element (a) of claim 1. Claims 10, 21, and 27 have been amended to delete the second occurrence of the compound "dioleoyl phosphatidylethanolamine (DOPE)" and to replace "." with —•— within the chemical formulas listed to more clearly indicate the association among the compounds. Claim 11 has been amended to correct improper claim dependency of the claim. Claim 1 provides antecedent basis for introducing nucleic acid molecules that encode a reporter gene into cells. Hence, claim 11, which further expands upon the types of nucleic acid molecules first introduced in claim 1, should depend on claim 1. No new matter has been added.

The specification is amended to correct obvious typographical, formatting and grammatical errors. The amendment to the paragraph beginning on page 8, line 15, through page 9, line 4, adds the inadvertently omitted phrase "of a nucleic acid molecule can occur" for grammatical clarity. The amendment finds basis in the specification at page 8, lines 15-21, which describes delivery of a

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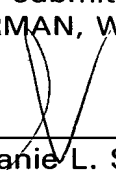
nucleic acid molecule. No new matter has been added.

Included as an attachment is a marked-up version of the specification paragraphs that are being amended.

\* \* \*

Entry of this amendment and examination of the application are respectfully requested.

Respectfully submitted,  
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# 12

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: *DE JONG et al.*

Serial No.: 10/086,745

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For: *METHODS FOR DELIVERING  
NUCLEIC ACID MOLECULES INTO  
CELLS AND ASSESSMENT  
THEREOF*

Art Unit: 1636

Examiner: Unassigned

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10/28/2002

Date

Michael Lough

**ATTACHMENT TO THE PRELIMINARY AMENDMENT  
MARKED UP PARAGRAPHS AND CLAIMS**

**IN THE SPECIFICATION:**

Please amend the specification as follows:

**Please amend the paragraph on page 4, lines 23-29, as follows:**

The delivery methods provided herein may also be used to deliver large nucleic acid molecules to a target cell [*in vitro*]*in vitro*, which is then introduced into an animal subject, in particular human subjects, such as may be done, for example, in a method of *ex vivo* gene therapy. Thus, also provided herein are methods of *in vivo* and *ex vivo* gene therapy using the methods for delivering large nucleic acid molecules into cells as provided herein.

**Please amend the paragraph beginning on page 8, line 15, through page 9, line 4, as follows:**

In particular embodiments, the methods of monitoring delivery and expression of a nucleic acid molecule include the steps of: introducing labelled nucleic acid molecules that encode a reporter gene into cells; detecting labelled cells as an indication of delivery of the nucleic acid into a cell; and measuring the product of the reporter gene as an indication of DNA expression in the cell, whereby delivery and expression of nucleic acid molecules in the cell is detected or determined. The labelled cells can be detected, for example, by flow cytometry, fluorimetry, cell imaging or fluorescence spectroscopy. The label, for example, can be iododeoxyuridine

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(IdU or IdUrd) or bromodeoxyuridine (BrdU), the reporter gene, for example, can be one that encodes fluorescent protein, enzyme, such as a luciferase, or antibody. The delivered nucleic acid molecules include, but are not limited to, RNA, including ribozymes, DNA, including naked DNA and chromosomes, plasmids, chromosome fragments, typically containing at least one gene or at least 1 Kb, naked DNA, or natural chromosomes. The method is exemplified herein by determining delivery and expression of artificial chromosome expression systems (ACes). Any types of cells, eukaryotic and prokaryotic, including cell lines, primary cells, primary cell lines, plant cells, and animal cells, including stem cells, embryonic cells, and other cells into which delivery of a nucleic acid molecule can occur, are [is] contemplated and may be used in the methods provided herein.

**Please amend the paragraph beginning on page 15, line 28, through page 16, line 14, as follows:**

As used herein, gene therapy involves the transfer or insertion of nucleic acid molecules, and, in particular, large nucleic acid molecules, into certain cells, which are also referred to as target cells, to produce specific gene products that are involved in correcting or modulating diseases or disorders. The nucleic acid is introduced into the selected target cells in a manner such that the nucleic acid is expressed and a product encoded thereby is produced. Alternatively, the nucleic acid may in some manner mediate expression of DNA that encodes a therapeutic product. This product may be a therapeutic compound, which is produced in therapeutically effective amounts or at a therapeutically useful time. It may also encode a product, such as a peptide or RNA, that in some manner mediates, directly or indirectly, expression of a therapeutic product. Expression of the nucleic acid by the target cells within an organism afflicted with a disease or disorder thereby [providing]provides a way to modulate the disease or disorder. The nucleic acid encoding the therapeutic product may be modified prior to introduction into the cells of the afflicted host in order to enhance or otherwise alter the product or expression thereof.

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Please amend the paragraphs beginning on page 16, line 19, through page 17, line 2, as follows:

As used herein, flow cytometry refers to processes that use a laser based instrument capable of analyzing and sorting out cells [and or]and/or chromosomes based on size and fluorescence.

As used herein, a reporter gene includes any gene that expresses a detectable gene product, which may be RNA or protein. Preferred reporter genes are those that are readily detectable. Examples of reporter genes include, but are not limited to nucleic acid encoding a fluorescent protein, CAT (chloramphenicol acetyl transferase) (Alton and Vapnek (1979), *Nature* 282: [864-869])864-869, luciferase, and other enzyme detection systems, such as beta-galactosidase; firefly luciferase (deWet *et al.* (1987), *Mol. Cell. Biol.* 7: 725-737); bacterial luciferase (Engebrecht and Silverman (1984), *PNAS* 1: 4154-4158; Baldwin *et al.* (1984), *Biochemistry* 23: 3663-3667); and alkaline phosphatase (Toh *et al.* (1989) *Eur. J. Biochem.* 182: 231-238, Hall *et al.* (1983) *J. Mol. Appl. Gen.* 2: 101).

Please amend the paragraph on page 20, lines 1-25, as follows:

**Cationic Compounds**

Cationic compounds for use in the methods provided herein are available commercially or can be synthesized by those of skill in the art. Any cationic compound may be used for delivery of nucleic acid molecules, such as DNA, into a particular cell type using the provided methods. One of skill in the art by using the provided screening procedures can readily determine which of the cationic compounds are best suited for delivery of specific nucleic acid molecules, such as DNA, into a specific target cell type.

**(a)Cationic Lipids**

Cationic lipid reagents can be classified into two general categories based on the number of positive charges in the lipid headgroup; either a single positive charge or multiple positive charges, usually up to 5.

Cationic lipids are often mixed with neutral lipids prior to use as delivery agents. Neutral lipids include, but are not limited to, lecithins; [phosphatidyl-ethanolamine]phosphatidylethanolamine; phosphatidylethanolamines, such as DOPE (dioleoylphosphatidylethanolamine), DPPE (dipalmitoylphosphatidylethanolamine), [dipalmitoylphosphatidylethanolamine,] POPE (palmitoyl-oleoyl-

phosphatidylethanolamine) and distearoylphosphatidylethanolamine; [phosphatidylcholine]phosphatidylcholine; phosphatidylcholines, such as DOPC ([dioleoylphosphatidylcholine]dioleoylphosphatidylcholine), DPPC (dipalmitoylphosphatidylcholine)[ POPC], POPC (palmitoylphosphatidylcholine) and distearoylphosphatidylcholine; fatty acid esters; glycerol esters; sphingolipids; cardiolipin; cerebrosides; and ceramides; and mixtures thereof. Neutral lipids also include cholesterol and other  $3\beta$ OH-sterols.

**Please amend the paragraphs beginning on page 21, line 1, through page 22, line 2, as follows:**

Examples of cationic lipid compounds include, but are not limited to: Lipofectin (Life Technologies, Inc., Burlington, Ont.)(1:1 (w/w) formulation of the cationic lipid N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and dioleoylphosphatidylethanolamine (DOPE)); LipofectAMINE (Life Technologies, Burlington, Ont., see U.S. Patent No. 5,334,761) (3:1 (w/w) formulation of polycationic lipid 2,3-dioleoyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA) and [dioleoyl phosphatidylethanolamine (DOPE)],dioleoylphosphatidylethanolamine (DOPE)); LipofectAMINE PLUS (Life Technologies, Burlington, Ont. see U.S. Patent Nos. 5,334,761 and 5,736,392; see, also U.S. Patent No. 6,051,429) (LipofectAmine and Plus [reagent],reagent); LipofectAMINE 2000 (Life Technologies, Burlington, Ont.; see also International PCT application No. WO 00/27795) (Cationic [lipid],lipid); Effectene (Qiagen, Inc., Mississauga, Ontario) (Non liposomal lipid [formulation],formulation); Metafectene (Biontex, Munich, Germany) (Polycationic [lipid],lipid); Eu-fectins (Promega Biosciences, Inc., San Luis Obispo, CA) (ethanolic cationic lipids numbers 1 through 12:  $[C_{52}H_{106}N_6O_4 \cdot 4CF_3CO_2H, C_{88}H_{178}N_8O_4S_2 \cdot 4CF_3CO_2H, C_{40}H_{84}NO_3P \cdot CF_3CO_2H, C_{50}H_{103}N_7O_3 \cdot 4CF_3CO_2H, C_{55}H_{116}N_8O_2 \cdot 6CF_3CO_2H, C_{49}H_{102}N_6O_3 \cdot 4CF_3CO_2H, C_{44}H_{89}N_5O_3 \cdot 2CF_3CO_2H, C_{100}H_{206}N_{12}O_4S_2 \cdot 8CF_3CO_2H, C_{162}H_{330}N_{22}O_9 \cdot 13CF_3CO_2H, C_{43}H_{88}N_4O_2 \cdot 2CF_3CO_2H, C_{43}H_{88}N_4O_3 \cdot 2CF_3CO_2H, C_{52}H_{106}N_6O_4 \cdot 4CF_3CO_2H, C_{88}H_{178}N_8O_4S_2 \cdot 4CF_3CO_2H, C_{40}H_{84}NO_3P \cdot CF_3CO_2H, C_{50}H_{103}N_7O_3 \cdot 4CF_3CO_2H, C_{55}H_{116}N_8O_2 \cdot 6CF_3CO_2H, C_{49}H_{102}N_6O_3 \cdot 4CF_3CO_2H, C_{44}H_{89}N_5O_3 \cdot 2CF_3CO_2H, C_{100}H_{206}N_{12}O_4S_2 \cdot 8CF_3CO_2H, C_{162}H_{330}N_{22}O_9 \cdot 13CF_3CO_2H, C_{43}H_{88}N_4O_2 \cdot 2CF_3CO_2H, C_{43}H_{88}N_4O_3 \cdot 2CF_3CO_2H, C_{41}H_{78}NO_8P]$ ); Cytofectene (Bio-Rad, Hercules, CA ) (mixture of a cationic lipid and a neutral [lipid],lipid); GenePORTER (Gene

Therapy Systems Inc., San Diego, CA) (formulation of a neutral lipid [(Dope)](DOPE) and a cationic lipid) and FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN) (Multi-component lipid based non-liposomal reagent).

**(b)Non-lipid cationic compounds**

Non-lipid cationic reagents include, but are not limited to SUPERFECT™ (Qiagen, Inc., Mississauga, ON) (Activated dendrimer (cationic polymer:charged amino [groups])groups) and CLONfectin™ (Cationic amphiphile N-t-butyl-N'-tetradecyl-3-tetradecyl-aminopropionamidine) (Clontech, Palo Alto, CA).

**Please amend the paragraph on page 34, lines 7-15, as follows:**

Also provided herein is a synoviocyte comprising a large heterologous nucleic acid, a heterologous chromosome or portion thereof, or an artificial chromosome. In one embodiment, the artificial chromosome is an ACes. Such synoviocytes include fibroblast-like synoviocytes. The synoviocytes may be from any species, including, but not limited to mammalian species. For example, synoviocytes containing large nucleic acids, such [as for example]as, for example, artificial chromosomes (*e.g.*, ACes) include primate synoviocytes, as well as rodent, rabbit, monkey and human synoviocytes.

**Please amend the paragraph on page 38, lines 16-26, as follows:**

**a.Transfer Efficiency**

A delivery method may be assessed by determining the percentage of recipient cells in which the nucleic acids, including DNA, [*is*]are present (*i.e.*, the transfer efficiency). However, when evaluating a delivery method for the ultimate goal of generating cells that express the transferred nucleic acid, there are additional factors beyond mere presence of the nucleic acid in recipient cells that should be considered. Included among these additional factors is cell viability. When assessing a proliferating cell population, clonogenicity is the method of choice to measure viability. When the target cells population is non-dividing or slow growing, metabolic integrity can be monitored.

**Please amend the paragraph on page 45, lines 23-31, as follows:**

Plasmid pIRES-EGFP was [*dervied*]derived from PIREsneo ([*originally*]originally called pCIN4) by replacing the neo gene downstream of the IRES sequence with the EGFP coding region. The IRES sequence permits translation of two open reading frames from one mRNA transcript. The

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expression cassette of pIRES-EGFP contains the human cytomegalovirus (CMV) major immediate early promoter/enhancer followed by a multiple cloning site (MCS), a synthetic intron (IVS; Huang *et al.* (1990) *Nucleic Acids Res.* 18: 937-947), the EMCV IRES followed by the EGFP coding region and the polyadenylation signal of bovine growth hormone.

**Please amend the paragraph on page 46, lines 22-31, as follows:**

**pCHEGFP2**

Plasmid pCHEGFP2 was constructed by deletion of the Nsi1/SmaI fragment from pIRES-EGFP. Plasmid pIRES-EGFP contains the coding sequence for a 2.1 kb [Nru 1/Xho] NruI/XhoI fragment of pCHEGFP2 containing the CMV promoter, synthetic intron, EGFP coding sequence and bovine growth hormone polyadenylation signal. Digestion of pIRES-EGFP with [Nru 1] NruI and [Sma 1] SmaI, yielded a 2.1 kb fragment. Digested DNA was fractionated by agarose gel electrophoresis, the separated band was excised and then eluted from the gel using the Qiaex 11 gel purification system (Qiagen, Mississauga, Ontario).

**Please amend the paragraph on page 50, lines 1-20, as follows:**

A9 cells were transfected using the Ca<sub>2</sub>PO<sub>4</sub> co-precipitation method (see, *e.g.*, Graham *et al.* (1978) *Virology* 52:456-457; Wigler *et al.* (1979) *Proc. Natl. Acad. Sci. U.S.A.* 76:1373-1376; and (1990) *Current Protocols in Molecular Biology*, Vol. 1, Wiley Inter-Science, Supplement 14, Unit 9.1.1-9.1.9). One day prior to transfection, A9 cells were plated at a density of 2 x 10<sup>6</sup> cells per 10 cm dish and 3 hours before transfection the medium was replaced with fresh growth medium. 140 µg of the 9 kb rDNA, *NotI* and 5 µg of the 2.1 kb CMV-EGFP [XhoI/NruI] XhoI/NruI fragments were mixed, co-precipitated and used to prepare the Ca<sub>2</sub>PO<sub>4</sub> co-precipitate (Calcium Phosphate Transfection System, (Canadian Life Technologies Burlington, [ON]) ON) which was distributed onto 2 10-cm dishes of subconfluent A9 cells. The DNA-Ca<sub>2</sub>PO<sub>4</sub> complexes were left on the cells for 18 hours, after which the precipitate was removed by aspiration and cells were subjected to glycerol shock for 1.5 minutes. After glycerol shock, the cell monolayers were gently washed with 2 X 10 ml of dPBS (Canadian Life Technologies Burlington, ON), followed by addition of 10 ml pre-warmed growth medium. Finally dishes were returned to the incubator and were maintained at 37°C, 5% CO<sub>2</sub>. After 3 hours recovery, each dish was passaged onto 3X 15 cm tissue dishes



Please amend the paragraphs beginning on page 53, line 3, through page 55, line 3, as follows:

For preparation of purified genomic DNA, sorted chromosome samples were brought to 0.5% SDS, 50 [mm]mM EDTA and 100  $\mu$ g/ml Proteinase K, then incubated for 18 hours at 50°C. 1  $\mu$ l of a 20 mg/ml glycogen solution (Boehringer Mannheim) was added to each sample, followed by extraction with an equal volume of Phenol: Chloroform: Isoamyl Alcohol (25:24:1). After centrifugation at 21,000Xg for 10 min, the aqueous phases were transferred to fresh microfuge tubes and were re-extracted as above. 0.2 volumes of 10 M  $\text{NH}_4\text{OAc}$ , 1  $\mu$ l of 20 mg/ml glycogen and 1 volume of iso-propanol were added to the twice extracted aqueous phases which were then vortexed and centrifuged for 15 minutes at 30,000Xg (at room [temperautre]temperature). Pellets were washed with 200  $\mu$ l of 70% ethanol and re-centrifuged as above. The washed pellets were air-dried then resuspended in 5mM Tris-Cl, pH 8.0 at  $0.5\text{-}2 \times 10^6$  chromosome equivalents/ $\mu$ l.

PCR was carried out on DNA prepared from sorted chromosome samples essentially as described (see, Co *et al.* (2000) *Chromosome Research* 8:183-191) using primers sets specific for EGFP and RAPSYN. Briefly, 50  $\mu$ l PCR reactions were carried out on genomic DNA equivalent to 10,000 or 1000 chromosomes in a solution containing 10 mM Tris-Cl, pH 8.3, 50mM KCl, 200  $\mu$ M dNTPs, 500 nM of forward and reverse primers, 1.5 mM  $\text{MgCl}_2$ , 1.25 units Taq polymerase (Ampli-Taq, Perkin-Elmer Cetus, CA). Separate reactions were carried out for each primer set. The reaction conditions were as follows: one cycle of 10 [min.]min at 95°C, then 35 cycles of 1 [min.]min at 94°C, 1 [min.]min at 55°C, 1 [min.]min at 72°C, and finally one cycle of 10 min at 72°C. After completion the samples were held at 4°C until analyzed by agarose gel electrophoresis using the following primers (SEQ ID Nos. 1-4, respectively):

EGFP forward primer 5'-cgtccaggagcgcaccatcttctt-3';

EGFP reverse primer 3'-atcgcgcttctcgttggggtcttt-3';

RAPSYN forward primer 5'-aggactgggtggctccaactcccagacac-3'; and

RAPSYN reverse primer 5'-agcttctcattgctgcgcgcagggttcagg-3'.

All primers were obtained from Canadian Life Technologies, Burlington, ON.

## EXAMPLE 2

### Preparation of Cationic vesicles

Vesicles were prepared at a lipid concentration of 700 [nmoles/ml]nmol/ml lipid (cationic lipid/DOPE 1:1) as follows. In a glass tube (10ml) 350 [nmoles]nmol cationic lipid (SAINT-2) was mixed with [350nmoles]350 nmol [dioleyl-phosphoethanolamine] dioleylphosphatidylethanolamine (DOPE), both solubilized in an organic solvent (Chloroform, Methanol or Chloroform/Methanol 1:1, v/v). [Diphosphatidyl-ethanolamine]Dioleylphosphatidylethanolamine (DOPE; Avanti Polar Lipids, Alabaster, AL) forms inverse hexagonal phases in a membrane and weakens the membrane. Other effectors that may be used are *cis*-unsaturated [phosphoethanolamines]phosphatidylethanolamines, *cis*-unsaturated fatty acids, and cholesterol. *Cis*-unsaturated phosphatidylcholines are less effective.

The solvent was evaporated under a stream of nitrogen (15 min/ 250  $\mu$ l solvent at room temperature). The remaining solvent was removed totally by drying the lipid for 15 min in [an]a desiccator under high vacuum from a vacuum pump. To the dried mixture was added [1 ml]1 ml ultrapure water. This was vortexed vigorously for about 5 min. The resulting solution was sonicated in an ultrasonication bath (Laboratory Supplies Inc. NY) until a clear solution was obtained. The resulting suspension contained a population of unilamellar vesicles with a size distribution between 50 to 100 nm.

## EXAMPLE 3

### Preparation of Cationic vesicles via alcoholic injection

In a glass tube (10ml) 350 [nmoles]nmol cationic lipid (Saint-2) was mixed with 350 [nmoles]nmol DOPE, both solubilized in an organic solvent (chloroform, methanol or chloroform/methanol 1/1). The solvent was evaporated under a stream of nitrogen (15 min/ 250  $\mu$ l solvent at room temperature). The remaining solvent was removed totally by drying the lipid for 15 min under high vacuum. This was then reconstituted in 100  $\mu$ l pure ethanol.

**Please amend the paragraphs on page 58, lines 2-25, as follows:**

After [incubation]incubation, Ultrasound gel (Other-Sonic Generic Ultrasound transmission gel, Pharmaceutical Innovations, Inc., Newark, NJ) was applied to the 2.5 cm sonoprotector head. Ultrasound was applied with an ImaRX Sonoprotector 100 at an output energy of 2.0 [Watt/cm<sup>2</sup>,] Watt/cm<sup>2</sup>, for 60 seconds, through the bottom of the plate of cells. After ultrasound of the well one chromosome per seeded cell ( $2 \times 10^5$ ) or 200  $\mu$ l GFP ACes in sheath buffer (15 mM Tris HCl, 0.1 mM EDTA, 20 mM NaCl, 1% hexylene glycol, 100 mM glycine, 20  $\mu$ M spermine and 50  $\mu$ M spermidine) are added immediately to the well. (Repeat until all samples on the plate requiring ultrasound have been treated). The plate was then sealed once more with parafilm tape and shaken gently (20 rpm) for 1 hour at room temperature.

After the [incubation]incubation, 1 ml (DMEM with 4500 mg/L D-glucose, L-glutamine and pyridoxine hydrochloride, 10% Fetal Bovine Serum, and a 1x solution of penicillin and streptomycin from a 10000 units/ml penicillin and 10000 mg/ml Streptomycin, 100x stock solution) was added to each well and the cells were incubated 18-24 hours at 37°C.

The cells in the plates were then washed with antibiotic containing medium and 2 ml of medium was placed in each well. The cells continued to be incubated at 37°C with 5% CO<sub>2</sub> until 48 hours after transfection/sonoporation. The cells were then [trypsinised]trypsinized and resuspended at a concentration of  $1 \times 10^6$  in DMEM to be analyzed by flow cytometry.

**Please replace the paragraphs beginning on page 59, line 8, through page 60, line 2, with the following:**

Transfection of the cells was performed as follows. The medium was removed from the cells, and the cells were washed twice with HBSS (Hanks balanced salt solution without Phenol Red (Gibco BRL, UK)) at 37°C. Then 500  $\mu$ l HBSS at 37°C was added per well, followed by 10  $\mu$ l of the freshly prepared vesicle solution (prepared in Example 2) to yield a final concentration of 23.3 [nmole/ml]nmol/ml.

Alternatively, the medium was removed from the cells, and the cells were washed twice with HBSS. 500  $\mu$ l HBSS/lipid solution at 37°C was added to each well. The HBSS/lipid solution was prepared by adding 1  $\mu$ l ethanolic lipid solution (prepared as described above) to 500  $\mu$ l HBSS under vigorous

vortexing. The plates were then sealed with parafilm tape and shaken gently at room temperature for 30 min. After incubation, ultrasound was applied at an output energy of 0.5 Watt/cm<sup>2</sup> for 60 sec through the bottom of the plate to the cells. The ultrasound was mediated by an ultrasound gel (Aquasonic 100, Parker, NJ) between transducer and plate. The ultrasound was applied with an ImaRx Sonoporator 100. Immediately after applying ultrasound one GFP chromosome per seeded cell ( $2 \times 10^5$  -  $5 \times 10^5$ ) (prepared in Example 1) was added. The plate was then sealed again and shaken gently for 1 h at room temperature. After the incubation 1ml medium (CHO-S-SFM 2 with 10% Fetal Calf Serum, 10000  $\mu$ g/ml Penicillin and 10000  $\mu$ g/ml Streptomycin Gibco BRL, Paisley, UK) was added to each well and the cells were incubated for 24 h at 37°C. The cells were then washed with [medium and]medium, 1 ml medium was [added]added, and the cells were incubated at 37° for another 24 h. Detection of expressed genes was then assayed by microscopy or detection of the transferred chromosome by FISH analysis.

**Please amend the paragraph on page 60, lines 11-17, as follows:**

**B. Ultrasound mediated transfection of Hep-G2 cells with Saint-2**

Hep-G2 cells were grown at 37°C, 5% CO<sub>2</sub>, in DMEM with 4500 mg/l Glucose, with [Pyridoxine/HCL,]Pyridoxine/HCL, 10% Fetal Calf Serum, 10000  $\mu$ g/ml Streptomycin and 1000  $\mu$ g/ml Penicillin. Between  $2 \times 10^5$  and  $5 \times 10^5$  cells were plated onto sterile glass slides in a 12 wells plate 24 hours before usage.

**Please amend the paragraph on page 60, lines 24-29, as follows:**

**C. Ultrasound mediated transfection of A9 cells with Saint-2**

A9 cells were grown at 37°C, 5% CO<sub>2</sub>, in DMEM with 4500 mg/l Glucose, with [Pyridoxine/HCL,]Pyridoxine/HCL, 10% Fetal Calf Serum, 10000  $\mu$ g/ml Streptomycin and 10000  $\mu$ g/ml Penicillin (GIBCO BRL, Paisley, UK). Between  $2 \times 10^5$  and  $5 \times 10^5$  cells were plated onto sterile glass slides in a 12 well plate 24 h before usage.

**Please amend the paragraphs beginning on page 65, line 8, through page 68, line 25, as follows:**

**EXAMPLE 9**

**A flow cytometry technique for measuring delivery of artificial chromosomes**

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Production cells lines (see Example 1) were grown in MEM medium (Gibco BRL) with 10% fetal calf serum (Can Sera, Rexdale ON) with 0.168  $\mu\text{g/ml}$  hygromycin B (Calbiochem, San Diego, CA). [~~Iododeoxyurine~~]Iododeoxyuridine or [~~Bromodeoxyuridine~~ were]Bromodeoxyuridine was added directly to culture medium of the production cell line (CHO E42019) in the exponential phase of growth. Stock [~~Iododeoxyuridine~~]Iododeoxyuridine was made in tris base pH 10. [~~Bromodeoxyuridine~~]10, Bromodeoxyuridine stocks in PBS. Final concentrations of 0.05-1  $\mu\text{M}$  for continuous label of 20-24 hours of 5-50  $\mu\text{M}$  with 15 minute pulse. After 24 hours, exponentially growing cells were blocked in mitosis with colchicine (1.0  $\mu\text{g/ml}$  for 7 hours before harvest. Chromosomes were then isolated and stained with Hoechst 33258 (2.5  $\mu\text{g/ml}$ ) and chromomycin A3 (50  $\mu\text{g/ml}$ ). Purification of artificial chromosomes was performed using a FACS Vantage flow cytometer (Becton Dickinson Immunocytometry systems, San Jose, CA). Chromomycin A3 was excited with the primary laser set at 457 nm, with emission detected using 475 nm long pass filter. Hoechst was excited by the secondary UV laser and emission detected using a 420/44 nm band-pass filter. Both lasers had an output of 150 mW. Bivariate distribution showing cell karyotype was accumulated from each sort. ACes were gated from other chromosomes and sorted. Condensing agents (hexylene glycol, spermine, and spermidine) were added to the sheath buffer to maintain condensed intact chromosome after sorting. IdU labeling index of sorted chromosomes was determined microscopically. [~~Aliquot~~]An aliquot (2-10  $\mu\text{l}$ ) of sorted chromosomes was fixed in 0.2% formaldehyde solution for 5 minutes before being dried on clean microscopic slide. [~~Microscope~~]The microscope sample was fixed with 70% ethanol. [~~Air-dried~~]The air-dried slide was denatured in coplin jar with [~~2NHCL~~]2 N HCl for 30 minutes at room temperature and washed 2-3 times with PBS. Non specific binding was blocked with PBS and 4% BSA or serum for minimum of 10 minutes. A 1/5 dilution of FITC conjugated IdU/BrdU antibody (Becton Dickinson) with a final volume of 60-100  $\mu\text{l}$  was applied to slide. Plastic strips, Durra seal (Diversified Biotech, Boston, [MA])MA, were overlaid on slides, and slides were kept in dark at 4°C in humidified covered box for 8-24 hours. DAPI (Sigma) 1  $\mu\text{g/ml}$  in Vectorshield was used as counterstain. Fluorescence was detected using Zeiss axioplan 2 microscope equipped for epifluorescence. [~~Minimum~~]A minimum of 100 chromosomes were

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scored for determining % labeled. Unlabeled chromosomes were used as negative control.

The day before the transfection, trypsinize V79-4 (Chinese Hamster Lung fibroblast) cells and plate at 250,000 into a 6 well petri dish in [4 mls]4 ml DMEM (Dulbecco's Modified Eagle Medium, Life Technologies) and 10% FBS (Can Sera Rexdale ON). The protocol was modified for use with LM (tk-) cell line by plating 500,000 cells. Lipid or dendrimer reagent was added to 1 X10<sup>6</sup> ACes sorted in ~800  $\mu$ l sort buffer. Exemplary protocol variations are set forth in Table 1. Chromosome and transfection agents were mixed gently. Complexes were added to cells drop-wise and plate swirled to mix. Plates were kept at 37°C in a 5% CO<sub>2</sub> incubator for specified transfection time. The volume in a well was then made up to 4-5 ml with DMEM and 10% FBS. Recipient cells were left for 24 hours at 37°C in a 5% CO<sub>2</sub> incubator. Trypsinize transfected cells. Samples to be analyzed for IdU labeled chromosome delivery are fixed in cold 70% ethanol and stored at -20°C, [tp]to be ready for IdU antibody staining. Samples to be grown for colony selection are counted and then transferred to 10-cm dishes at densities of 10,000 and 100,000 cells in duplicate with remaining cells put in a 15 cm dish. After 24 hours, selection medium containing of DMEM and 10% FBS with 0.7 mg/ml hygromycin B, # 400051 (Calbiochem San Diego, CA) is added. Selection medium is changed every 2-3 days. This concentration of hygromycin B kills the wild type cells after selection for 7 days. At 10-14 days colonies were expanded and then screened by FISH for intact chromosome transfer and assayed for beta galactosidase expression.

**Table 1: Delivery Transfection Protocols**

Agent	Dilution Stock	Pre treatment of ACes	Complexing time (minutes)	Added to complexes	Medium (ml) added to wells before complexes	Transfection time (hours)
CLONFECTIN	2-8 $\mu$ g in NaCl-HEPES		20	1.8 ml of serum free		4
CYTOFECTENE			10-20	200 $\mu$ l of 50% FBS plus DMEM		24

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Agent	Dilution Stock	Pre treatment of ACes	Complexing time (minutes)	Added to complexes	Medium (ml) added to wells before complexes	Transfection time (hours)
ENHANCER + EFFECTENE (1:5 ratio)		Enhancer 5 minutes	10		1.2	3
EU-FECTIN-1 to 11			5-10			6
FUGENE 6	0.5-6 $\mu$ l to final volume of 100 $\mu$ l in serum free medium		15-45			4
GENEPORTER 2	2.5 $\mu$ l added to 150 $\mu$ l of serum free medium		2-10			2-4
LIPOFECTAMINE			15			3
LIPOFECTAMINE 2000			20		2.5	5
METAFECTENE	diluted into 60 $\mu$ l serum free medium		15-45		0.8	6
PLUS + LIPOFECTAMINE (1:1 and 3:2 ratio)		PLUS and 200 $\mu$ l of DMEM for 15 minutes	15			3
SUPERFECT			10		0.6	3

**[IDU]IdU ANTIBODY LABELING**

A standard BrdU staining flow cytometry protocol (Gratzer et al. Cytometry (1981);6:385-393) was used except with some modifications at the neutralization step, the presence of detergent during denaturation and the composition of blocking buffer. Between each step samples are centrifuged at 300 g for 7-10 minutes and supernatant removed. Samples of 1-2 million cells are fixed in 70% cold ethanol. Cells are then denatured in 1-2 ml of 2N HCL plus 0.5% triton X for 30 minutes at room temperature. Sample undergoes 3-4

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washes with cold DMEM until indicator is neutral. Final wash with cold DMEM plus 5% FBS. Blocking/permeabilization buffer containing PBS, 0.1% triton X and 4% FBS is added for 10-15 minutes before pelleting sample by centrifugation. Add 20  $\mu$ l of IdU/BrdU FITC conjugated B44 clone antibody (Becton Dickinson Immunocytometry Systems, San Jose, CA) to pellet and leave for 2 hours at room temperature in the dark with agitation every 30 minutes. Wash cells with block/permeabilization buffer and resuspend in PBS for flow analysis.

**FLOW CYTOMETRY DETECTION OF FLUORESCENT [IDUrdD]IdUrd  
LABELED ACes**

Percentage of transfected cells containing IdU labeled ACes was determined using a flow cytometry with an argon laser turned to 488 nm at 400 mW. FITC fluorescence was collected through a standard FITC 530/30-nm band pass filter. Cell populations were gated on the basis of side scatter versus forward scatter to exclude debris and doublets. Data was accumulated (15,000 events) to form bivariate channel distribution showing forward scatter versus green fluorescence (IdU-FITC). The fluorescence level at which cells were determined to be positive was established by visual inspection of the histogram of negative control cells, such that approximately 1% appeared in the positive region.

**IN THE CLAIMS:**

Please amend claims 1, 9-11, 21, and 27 as follows:

1. (Amended) A method for detecting or determining delivery and expression of nucleic acid introduced into a cell comprising;

(a) introducing labelled nucleic acid molecules that encode a reporter gene into cells;

(b) detecting labelled cells as an indication of delivery of the nucleic acid into a cell; and

(c) measuring the product of the reporter gene as an indication of DNA expression in the cell, whereby delivery and expression of nucleic acid molecules in the cell is detected or determined.

9. (Amended) The method of claim [16,]1, wherein step (a) further comprises contacting the nucleic acid molecule is with a delivery agent that comprises a cationic compound.



10. (Amended) The method of claim 9, wherein the compound is selected from the group consisting of N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), dioleoylphosphatidylethanolamine (DOPE), 2,3-dioleoyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), [dioleoyl phosphatidylethanolamine (DOPE),  $C_{52}H_{106}N_6O_4 \cdot 4CF_3CO_2H$ ,  $C_{88}H_{178}N_8O_4S_2 \cdot 4CF_3CO_2H$ ,  $C_{40}H_{84}NO_3P \cdot CF_3CO_2H$ ,  $C_{50}H_{103}N_7O_3 \cdot 4CF_3CO_2H$ ,  $C_{55}H_{116}N_8O_2 \cdot 6CF_3CO_2H$ ,  $C_{49}H_{102}N_6O_3 \cdot 4CF_3CO_2H$ ,  $C_{44}H_{89}N_5O_3 \cdot 2CF_3CO_2H$ ,  $C_{41}H_{78}NO_8P$ ),  $C_{100}H_{206}N_{12}O_4S_2 \cdot 8CF_3CO_2H$ ,  $C_{162}H_{330}N_{22}O_9 \cdot 13CF_3CO_2H$ ,  $C_{43}H_{88}N_4O_2 \cdot 2CF_3CO_2H$ ,  $C_{43}H_{88}N_4O_3 \cdot 2CF_3CO_2H$ ]  $C_{52}H_{106}N_6O_4 \cdot 4CF_3CO_2H$ ,  $C_{88}H_{178}N_8O_4S_2 \cdot 4CF_3CO_2H$ ,  $C_{40}H_{84}NO_3P \cdot CF_3CO_2H$ ,  $C_{50}H_{103}N_7O_3 \cdot 4CF_3CO_2H$ ,  $C_{55}H_{116}N_8O_2 \cdot 6CF_3CO_2H$ ,  $C_{49}H_{102}N_6O_3 \cdot 4CF_3CO_2H$ ,  $C_{44}H_{89}N_5O_3 \cdot 2CF_3CO_2H$ ,  $C_{41}H_{78}NO_8P$ ,  $C_{100}H_{206}N_{12}O_4S_2 \cdot 8CF_3CO_2H$ ,  $C_{162}H_{330}N_{22}O_9 \cdot 13CF_3CO_2H$ ,  $C_{43}H_{88}N_4O_2 \cdot 2CF_3CO_2H$ ,  $C_{43}H_{88}N_4O_3 \cdot 2CF_3CO_2H$  and (1-methyl-4-(1-octadec-9-enyl-nonadec-10-enylenyl)pyridinium chloride.

11. (Amended) The method of claim [10]1, wherein the nucleic acid molecules are natural chromosomes, artificial chromosomes, fragments of a chromosome or naked DNA that is greater than at least about 0.6 megabase in size.

21. (Amended) The method of claim 20, wherein the compound is selected from the group consisting of N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), dioleoylphosphatidylethanolamine (DOPE), 2,3-dioleoyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), [dioleoyl phosphatidylethanolamine (DOPE),  $C_{52}H_{106}N_6O_4 \cdot 4CF_3CO_2H$ ,  $C_{88}H_{178}N_8O_4S_2 \cdot 4CF_3CO_2H$ ,  $C_{40}H_{84}NO_3P \cdot CF_3CO_2H$ ,  $C_{50}H_{103}N_7O_3 \cdot 4CF_3CO_2H$ ,  $C_{55}H_{116}N_8O_2 \cdot 6CF_3CO_2H$ ,  $C_{49}H_{102}N_6O_3 \cdot 4CF_3CO_2H$ ,  $C_{44}H_{89}N_5O_3 \cdot 2CF_3CO_2H$ ,  $C_{41}H_{78}NO_8P$ ),  $C_{100}H_{206}N_{12}O_4S_2 \cdot 8CF_3CO_2H$ ,  $C_{162}H_{330}N_{22}O_9 \cdot 13CF_3CO_2H$ ,  $C_{43}H_{88}N_4O_2 \cdot 2CF_3CO_2H$ ,  $C_{43}H_{88}N_4O_3 \cdot 2CF_3CO_2H$ ]  $C_{52}H_{106}N_6O_4 \cdot 4CF_3CO_2H$ ,  $C_{88}H_{178}N_8O_4S_2 \cdot 4CF_3CO_2H$ ,  $C_{40}H_{84}NO_3P \cdot CF_3CO_2H$ ,  $C_{50}H_{103}N_7O_3 \cdot 4CF_3CO_2H$ ,  $C_{55}H_{116}N_8O_2 \cdot 6CF_3CO_2H$ ,  $C_{49}H_{102}N_6O_3 \cdot 4CF_3CO_2H$ ,  $C_{44}H_{89}N_5O_3 \cdot 2CF_3CO_2H$ ,  $C_{41}H_{78}NO_8P$ ,  $C_{100}H_{206}N_{12}O_4S_2 \cdot 8CF_3CO_2H$ ,  $C_{162}H_{330}N_{22}O_9 \cdot 13CF_3CO_2H$ ,  $C_{43}H_{88}N_4O_2 \cdot 2CF_3CO_2H$ ,  $C_{43}H_{88}N_4O_3 \cdot 2CF_3CO_2H$  and (1-methyl-4-(1-octadec-9-enyl-nonadec-10-enylenyl)pyridinium chloride.

27. (Amended) The method of claim 26, wherein the compound is selected from the group consisting of N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), dioleoylphosphatidylethanolamine (DOPE), 2,3-dioleoyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), [dioleoyl phosphatidylethanolamine (DOPE),  $C_{52}H_{106}N_6O_4 \cdot 4CF_3CO_2H$ ,  $C_{88}H_{178}N_8O_4S_2 \cdot 4CF_3CO_2H$ ,  $C_{40}H_{84}NO_3P \cdot CF_3CO_2H$ ,  $C_{50}H_{103}N_7O_3 \cdot 4CF_3CO_2H$ ,  $C_{55}H_{116}N_8O_2 \cdot 6CF_3CO_2H$ ,  $C_{49}H_{102}N_6O_3 \cdot 4CF_3CO_2H$ ,  $C_{44}H_{89}N_5O_3 \cdot 2CF_3CO_2H$ ,  $C_{41}H_{78}NO_8P$ ),  $C_{100}H_{206}N_{12}O_4S_2 \cdot 8CF_3CO_2H$ ,  $C_{162}H_{330}N_{22}O_9 \cdot 13CF_3CO_2H$ ,  $C_{43}H_{88}N_4O_2 \cdot 2CF_3CO_2H$ ,  $C_{43}H_{88}N_4O_3 \cdot 2CF_3CO_2H$ ]  $C_{52}H_{106}N_6O_4 \cdot 4CF_3CO_2H$ ,  $C_{88}H_{178}N_8O_4S_2 \cdot 4CF_3CO_2H$ ,  $C_{40}H_{84}NO_3P \cdot CF_3CO_2H$ ,  $C_{50}H_{103}N_7O_3 \cdot 4CF_3CO_2H$ ,  $C_{55}H_{116}N_8O_2 \cdot 6CF_3CO_2H$ ,  $C_{49}H_{102}N_6O_3 \cdot 4CF_3CO_2H$ ,  $C_{44}H_{89}N_5O_3 \cdot 2CF_3CO_2H$ ,  $C_{41}H_{78}NO_8P$ ,  $C_{100}H_{206}N_{12}O_4S_2 \cdot 8CF_3CO_2H$ ,  $C_{162}H_{330}N_{22}O_9 \cdot 13CF_3CO_2H$ ,  $C_{43}H_{88}N_4O_2 \cdot 2CF_3CO_2H$ ,  $C_{43}H_{88}N_4O_3 \cdot 2CF_3CO_2H$  and (1-methyl-4-(1-octadec-9-enyl-nonadec-10-enylenyl)pyridinium chloride.